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(54) Title: METHOD AND COMPOSITIONS FOR SELECTIVELY INHIBITING AMPLIFICATION OF SEQUENCES IN A POPULATION OF NUCLEIC ACID MOLECULES

(57) Abstract: Disclosed are methods and compositions for selectively inhibiting the amplification of a desired sequence in a population of nucleic acid sequences.

METHOD AND COMPOSITIONS FOR SELECTIVELY INHIBITING AMPLIFICATION OF SEQUENCES IN A POPULATION OF NUCLEIC ACID MOLECULES

FIELD OF THE INVENTION

The invention relates to methods and compositions for selectively inhibiting amplification of undesired sequences in a population of nucleic acid molecules.

BACKGROUND OF THE INVENTION

Approximately 10,000-20,000 genes are believed to be expressed within living cells, depending upon the specific cell type. RNAs corresponding to different genes can be present in different levels in cells. For example, transcripts from as few as 10-15 genes may represent 10-15% of cellular mRNA by mass. In addition to these highly abundant transcripts, another 1000-2000 genes encode moderately abundant transcripts, which can account for up to 50% of cellular mRNA mass. Transcripts from the remaining genes fall into the low abundance class.

Because many genes are identified by isolating complementary DNA (cDNA) corresponding to an RNA sequence, a significant problem can arise because of differences in the levels at which specific RNAs are present in cell types. The most abundant sequences can be repeatedly sampled, while the lowest abundance class may be rarely, if ever, sampled.

Several normalization and subtractive hybridization protocols have been developed to help overcome this problem. These techniques can be technically difficult to perform, and they can fail to detect cDNAs corresponding to rare transcripts.

SUMMARY OF THE INVENTION

The invention is based in part on the discovery of a method for easily and inhibiting the amplification of repetitive nucleic acid sequences in a population of nucleic acids.

The invention features a method of selectively inhibiting amplification of a target nucleic acid in a population of nucleic acid molecules. The method includes providing a population of nucleic acid molecules and contacting the population of nucleic acid molecules with at least one blocking primer to form an annealed blocking primer- template complex that includes the blocking primer and a complementary target sequence in the population of nucleic acid molecules. The blocking primer cannot be extended with a polymerase.

An extendable primer is also contacted with the population of nucleic acid molecules under conditions that allow for formation of an annealed extendable primer-template complex. The extendable-primer template complex with a polymerase. However, the polymerase does not extend the extended blocking primer template complex. Thus, amplification of the target nucleic acid can be selectively inhibited.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration comparing the structure of a peptide nucleic acid (PNA) and a deoxyribonucleic acid (DNA) molecule.

FIG. 2 is a schematic illustration showing the alignment of the PNA oligonucleotides and restriction fragments of ISGF-3B cDNA.

FIG. 3 is a representation of a graph showing the amounts of amplified sequence (y-axis) as a function of the size of the sequence product (x-axis) in the presence of various amounts of PNA oligomers.

DETAILED DESCRIPTION OF THE INVENTION

Amplification of a specific nucleic acid in a population has been inhibited by including a non-extendable blocking primer in the amplification. The non-extendible blocking primer hybridizes to the target nucleic acid but is not extended by a polymerase. The blocking primers described herein can also be used to inhibit replication and/or transcription of desired RNA sequences.

A preferred blocking primer is a peptide nucleic acid (PNA) oligomers. PNA oligomers are analogs of DNA in which the phosphate backbone is replaced with a peptide-like backbone. The structure of PNAS and conventional nucleic acids are shown schematically in FIG. 1. The achiral backbone of a the PNA oligomer is made from N-(2-aminoethyl)-glycine units linked by amide bonds. The backbone is uncharged. The four standard monomers A, C, G, and T are attached to the backbone by methylene carbonyl linkages (where B = base). The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup, *et al.*, 1996. *Bioorg. Med. Chem.* 4: 5-23, and Perry-O'Keefe, *et al.*, 1996. *Proc. Natl. Acad. Sci. USA* 93: 14670-14675.

PNAs have been shown to bind to their complementary nucleic acid sequences with greatly improved affinity and specificity compared to DNA. In addition, the

thermal stability of a PNA/DNA or PNA/RNA duplex is essentially independent of the salt concentration in the hybridization solution, and can be used under low-salt conditions that cause the target nucleic acid sequence to unwind. Because PNA oligomers lack a phosphate backbone, PNA-oligomers also block the extension of cDNA synthesis mediated by reverse transcriptase, and thus inhibit the first strand cDNA synthesis from the respective mRNA.

The blocking primers can be used to selectively inhibit amplification of a target nucleic acid in a population of nucleic acids by annealing the blocking primer to a target nucleic acid in the population of nucleic acids. Annealing of the blocking primer to the template results in formation of a double-stranded blocking primer- template complex that includes the blocking primer and the region of the target nucleic acid to which the blocking primer is bound.

An extendable primer is also annealed to the population of nucleic acids to form an annealed extendable primer-template complex. By extendable primer is meant that the nucleotides can be incorporated onto the end of the annealed primer in the presence of a polymerase, nucleotide triphosphates, and other cofactors. Because the blocking primer is not extendable, the polymerase does not extend extended blocking primer template complex. Thus, amplification of the target sequence hybridizing to the blocking primer can be selectively inhibited.

The particular target sequence whose amplification is amplified is determined by the binding specificity of the blocking primer. Thus, any desired sequence can be selectively inhibited, as long as it is possible to design a blocking primer that binds specifically to the target sequence. In some embodiments, the target nucleic acid is present at relatively high copy number in the population of nucleic acids. For example the blocking primer can be designed to hybridize to moderately or highly abundant transcripts. Alternatively, or in addition, the blocking primer can be designed to hybridize to a sequence whose amplification is otherwise not desired, *e.g.*, because it has been previously characterized.

In various embodiments, the blocking primer is between 8 and 50 nucleotides in length, *e.g.*, the blocking primer is between about 10 and 30 nucleotides or 13 and 30 nucleotides in length.

The blocking primer can be provided at a concentration of about 50 picomoles/μ l to about 700 picomoles/μ l, *e.g.*, the blocking primer can be provided at a concentration of about 100 picomoles/μ l to about 500 picomoles/μ l or about 250picomoles/μ l to about 350 picomoles/μ l.

In preferred embodiments, more than one blocking primer is used. In some embodiments, the blocking primer anneals to a region of a target nucleic acid that is physically linked to the binding region of a first blocking primer. For example, multiple blocking primers can be designed to anneal to a specific RNA molecule. Preferably, one or more of the blocking primers anneal to a region complementary to the 3' and of the RNA molecule.

In other embodiments, an additional blocking primer is designed to anneal to a target nucleic acid that corresponds to the 5' region of the second strand synthesized in a mRNA to cDNA based amplification. For example, inhibition of a specific mRNA may be desired. The first blocking primer anneals to a sequence near the 3' end of the mRNA molecule. The second blocking primer anneals to a sequence that binds to a sequence homologous to the 5' end of the RNA.

In preferred embodiments, the blocking primer anneals to a target sequence near the end (*e.g.*, the 3' end) of a nucleic acid molecule.

While the blocking primer or primers of the invention can be used in conjunction with a single extendable primer, in some embodiments multiple extendable primers can be used.

Any desired population can be used as the source of the population of nucleic acid molecules. Thus, the nucleic acid can be a genomic DNA, a cDNA, or an mRNA (such as polyA+ RNA). When RNA is used it can be derived from, *e.g.*, a plant, a

single-celled animal, a multi-cellular animal, a bacterium, a virus, a fungus, or a yeast. If desired, the RNA can also be partitioned prior to use with a blocking primer

When poly A+ containing RNA is used, a preferred blocking primer is one that includes at its 5' terminus an oligo dT sequence, and at its 3' terminus a sequence that is specific for a polyA+-containing RNA of interest.

In general, any nucleic acid polymerase that can extend the annealed extendable primer. Suitable polymerases include, *e.g.*, DNA-dependent DNA polymerases, RNA-dependent DNA polymerases (reverse transcriptases), DNA-dependent RNA polymerases, and RNA-dependent RNA polymerases.

Examples of DNA-dependent DNA polymerases include, *e.g.*, the DNA polymerase from *Bacillus stearothermophilus* (Bst), the *E. coli* DNA polymerase I Klenow fragment, the bacteriophage T4 and T7 DNA polymerases, and those from *Thermus aquaticus* (Taq), *Pyrococcus furiosis* (Pfu), and *Thermococcus litoralis* (Vent). The Bst DNA polymerase has been shown to efficiently incorporate 3'-O-(-2-Nitrobenzyl)-dATP into a growing DNA chain, is highly processive, very stable, and lacks 3'-5' exonuclease activity. The coding sequence of this enzyme has been determined. See U.S. Patent Nos. 5,830,714 and 5,814,506, incorporated herein by reference.

Examples of reverse transcriptases include, *e.g.*, reverse transcriptase from Avian Myeloblastosis Virus (AMV), Moloney Murine Leukemia Virus, and Human Immunodeficiency Virus-1 (HIV-1). HIV-1 reverse transcriptase is particularly preferred because it is well characterized both structurally and biochemically. See, *e.g.*, Huang, *et al.*, *Science* **282:** 1669-1675 (1998).

A suitable DNA-dependent RNA polymerase can be used when an RNA product is desired. Preferred examples of these enzymes include, *e.g.*, RNA polymerase from *E. coli* [Yin, *et al.*, *Science* ***270***: 1653-1657 (1995)] and RNA polymerases from the bacteriophages T7, T3, and SP6. Alternatively, a modified T7 RNA polymerase functions as a DNA dependent DNA polymerase.

Suitable RNA-dependent RNA polymerases include, *e.g.*, RNA-dependent RNA polymerases from the viral families: bromoviruses, tobamoviruses, tombusvirus, leviviruses, hepatitis C-like viruses, and picornaviruses. See, *e.g.*, Huang *et al.*, *Science* **282**: 1668-1675 (1998); Lohmann *et al.*, *J. Virol.* **71**: 8416-8428 (1997); Lohmann *et al.*, *Virology* **249**:108-118 (1998), and O'Reilly and Kao, *Virology* **252**: 287-303 (1998).

The invention will be further illustrated in the following non-limiting examples.

Example 1: Inhibition of cDNA synthesis using PNA oligonucleotides

To demonstrate inhibition of cDNA synthesis using PNA oligonucleotides, PNA oligomers specific for transcription factor ISGF-3B mRNA were synthesized. The synthesized included a 15-mer at position 116-130 for sense for the sense strand and a 16 mer at position 241-256 for the antisense stand. The PNA oligonucleotides were used to inhibit the first strand cDNA synthesis of poly A+ mRNA isolated from human MG-63 cells.

The two biotinylated 15-mer PNA-oligomers were purchased from PE Biosystems (PE Biosystems, 500 Old Connecticut Path, Framingham, MA 01701). The sequence of the sense PNA-oligomer was CAG TCT TGG CAC CTA (SEQ ID NO:1) (position 116-130), and antisense PNA-oligomer CTG GTG AAC CTG CTC (SEQ ID NO:2) (position 241-256). The concentrations were adjusted to 100 pmoles/μl with ddH_2O and stored in aliquots at –20°C until used.

To isolate polyA+ RNA, human MG-63 cells were lysed, and total cellular mRNA was isolated according using a trizol lysate procedure (Life Technologies, Rockville, MD), except that the genomic DNA digestion step using DNAse was omitted. After quantitation using a GeneQuant photometer, total RNA was processed directly for poly A+ isolation using a CPG-Strepavidin mRNA isolation kit (CPG, Inc.,

Lincoln Park, NJ). The estimated yield of total RNA from 6 T150 flasks of MG-63 cells was 580 μg. Poly A+ was stored in aliquots at –20°C in 70% ethanol until used.

To perform cDNA synthesis in the presence of PNA poisoning primers, two aliquots of poly A+ mRNA (from ~230 μg total RNA) were precipitated, pooled and dissolved in 24 μl oligo-dT solution (100 pmoles/ μl), and distributed equally into 12 well strips of PCR thin wall tubes. PNAs (100 pmoles/ μl) and DEPC-H_2O were added according to following table:

Sample	DEPC-H_2O	Antisense PNA	Sense PNA
1 and 2	10 μl	0 μl	0 μl
3 and 4	8 μl	1 μl	1 μl
5 and 6	6 μl	2 μl	2 μl
7 and 8	4 μl	3 μl	3 μl
9 and 10	2 μl	4 μl	4 μl
11 and 12	0 μl	5 μl	5 μl

Samples were heated to 70°C in a thermocycler for 10 min and transferred to an ice bath for 2 min. Subsequent steps and second-strand synthesis were performed essentially as described in Gubler et al., Gene 25:263,1983. Quantitation of cDNA yield was performed using PicoGreen fluorometry. Final cDNA concentration of all samples was adjusted to 1 ng/ μl with TE buffer and stored at –20°C until used.

Quantitative Expression Analysis (QEA) reactions as described in US Patent No. 5,871,697 and in Shimkets at al. Nat Biotechnol. 1999 Aug;17(8):798-803 were prepared for sequences named as follows: d0h1 (172 nt), d0p0 (71 nt), d0y0 (370 nt), m1s0 (267 nt), s0c0 (104 nt), s0x1 (253 nt), y0h0 (141 nt) and l0m0 (349.8 nt). FIG.2 shows the location of the PNA oligomers on the ISGF-3B cDNA construct (denoted as "PNA002" and "PNA001"). Also shown are sequence fragments denoted 10m0-349.8 and d0yo-70 from this region.

A mixture of both sense and antisense IGSF-3B PNA-oligomers dose dependently and specifically inhibited the first strand cDNA synthesis of the IGSF-3B gene. The inhibitory effect on amplification of the 10m0-349.8 and d0y0 fragments is shown in FIGS. 3A and 3B. The figures show that as increasing amounts of the PNA oligomers were added, the size of the peak corresponding to 10m0 349.8 (FIG. 3A) or d0y0 370 (FIG. 3B) decreased. Since the PNAs were designed to inhibit the subsequence l0m0 349.8 which is located at the 5' end of ISGF-3B gene, the most complete inhibition was observed for this subsequence . The reverse transcription of downstream fragments appeared unaffected. This suggests that the PNA oligomers specifically inhibit amplification of nucleic acid sequences.

What is claimed is:

1. A method of selectively inhibiting amplification of a target nucleic acid in a population of nucleic acid molecules, the method comprising

providing a population of nucleic acid molecules;

contacting said population of nucleic acid molecules with at least one first blocking primer to form an annealed blocking primer- template complex, wherein said first blocking primer is complementary to a target nucleic acid in said population of nucleic acid molecules;

contacting said population with at least one extendable primer to form an annealed extendable primer-template complex;

and

extending said annealed extendable-primer template complex with a polymerase, wherein said polymerase does not extend said extended blocking primer template complex,

thereby selectively inhibiting amplification of said target nucleic acid.

2. The method of claim 1, wherein said target nucleic acid is present at high copy number in the population of nucleic acid molecules.

3. The method of claim 1, wherein said blocking primer is a peptide-nucleic acid.

4. The method of claim 3, wherein said blocking primer is between 8 and 50 nucleotides in length.

5. The method of claim 3, wherein said blocking primer is between about 10 and 30 nucleotides in length.

6. The method of claim 3, wherein said blocking primer is between 13 and 20 nucleotides in length.

7. The method of claim 3, wherein said blocking primer is provided at a concentration of about 50 picomoles/μ l to about 700 picomoles/μ l.

8. The method of claim 3, wherein said blocking primer is provided at a concentration of about 100 picomoles/μ l to about 500 picomoles/μ l.

9. The method of claim 3, wherein said blocking primer is provided at a concentration of about 250 picomoles/μ l to about 350 picomoles/μ l.

10. The method of claim 1, further comprising contacting the population of nucleic acid molecules with a second blocking primer.

11 The method of claim 1, further comprising contacting the population of nucleic acid molecules with a second extendable primer.

12. The method of claim 1, wherein said population of nucleic acid molecules is RNA.

13. The method of claim 12, wherein said population of RNA molecules is polyA+ RNA.

14. The method of claim 1, wherein the first extendable primer comprises a 3' terminal oligo dT sequence.

15. The method of claim 1, wherein said polymerase is an RNA directed DNA polymerase.

16. The method of claim 1, further comprising contacting said population of nucleic acid molecules with a second blocking primer.

17. The method of claim 16, further comprising contacting said population of nucleic acid molecules with third blocking primer..

18. A method of selectively inhibiting amplification of a target nucleic acid in a population of RNA molecules, the method comprising

providing a population of RNA molecules;

contacting said population of nucleic acid molecules with a blocking primer;

contacting said population with an extendable primer comprising extendable 3' terminal oligo dT sequence to form an annealed extendable primer-template complex;

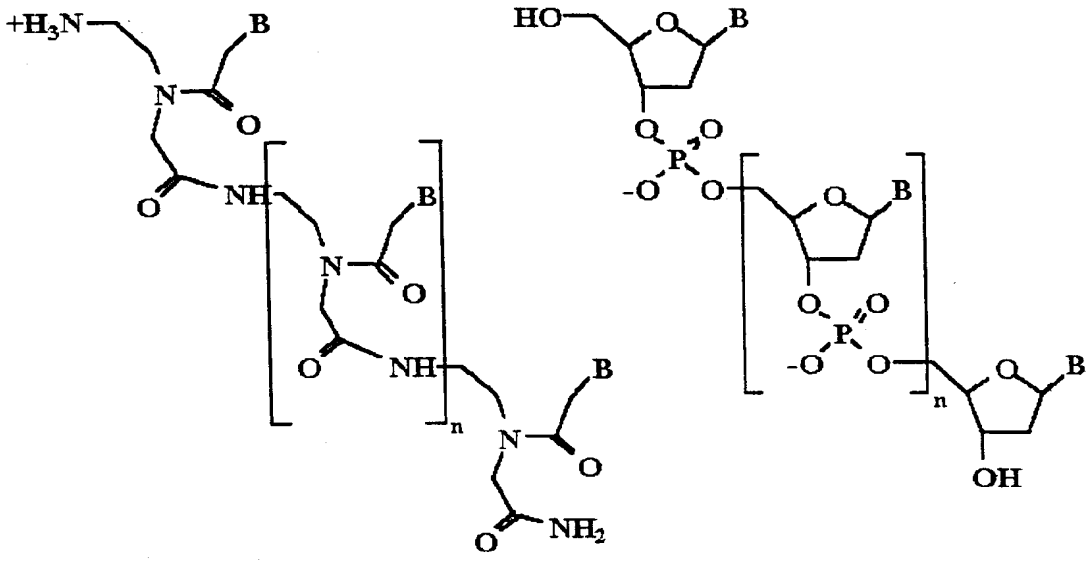
and

extending said extendable primer complex with a RNA-directed DNA polymerase, wherein said polymerase does not extend said extended blocking primer template complex,

thereby selectively inhibiting amplification of said target nucleic acid.

19. The method of claim 18, wherein said blocking primer is a peptide nucleic acid.

20. The method of claim 18, further comprising a second blocking primer.



PNA DNA

FIG. 1

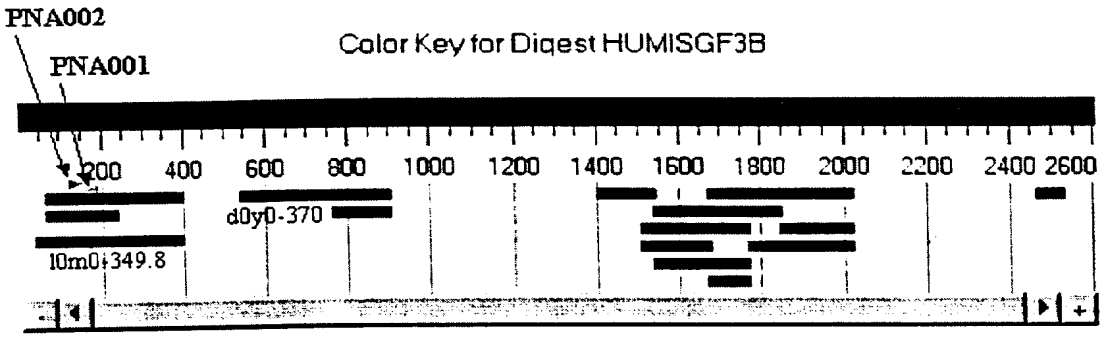

PNA002
PNA001
Color Key for Digest HUMISGF3B
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
d0y0-370
10m0+349.8

FIG. 2

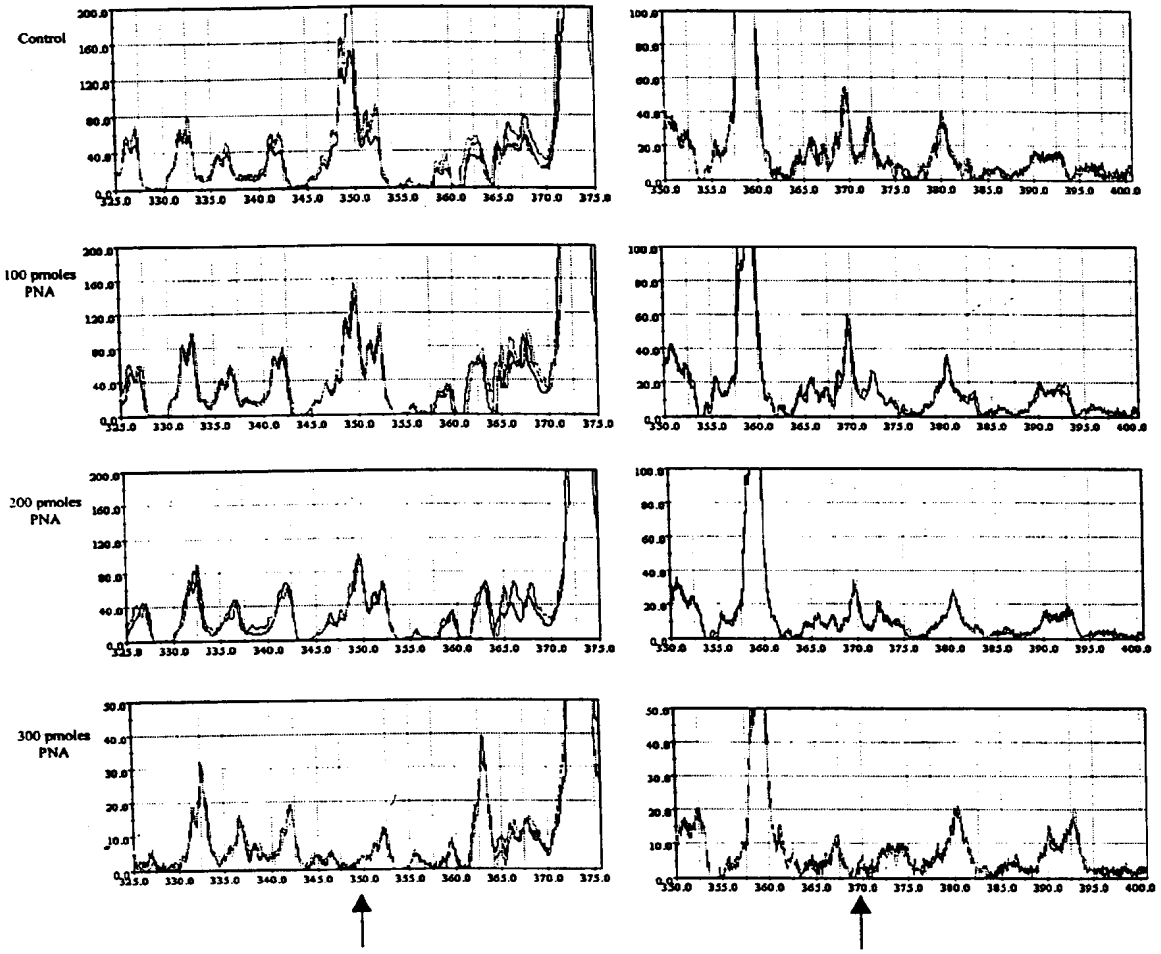


Fig. 3A: QEA fragment l0m0 349.8

Fig. 3B: QEA fragment d0y0 370